Differences in substrate and inhibitor sequence specificity of human, mouse and rat tissue kallikreins

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The kininogenase activities of mouse (mK1), rat (rK1) and human (hK1) tissue kallikreins were assayed with the bradykinin-containing synthetic peptides Abz-MTEMARRPPGFSPFRSVTVQ-NH_2 (where Abz stands for *o*-aminobenzoyl) and Abz-MTSVIRRPPGFSPFRAPRV-NH_2, which correspond to fragments Met^{374}-Gln^{393} and Met^{375}-Val^{393} of mouse and rat LMWKs (low-molecular-mass kininogens) with the addition of Abz. Bradykinin was released from these peptides by the mK1- and rK1-mediated hydrolysis of Arg–Arg and Arg–Ser (or Arg–Ala) peptide bonds. However, owing to preferential hydrolysis of Phe–Arg compared with the Arg–Ala bond in the peptide derived from rat LMWK, hK1 released bradykinin only from the mouse LMWK fragment and preferentially released des-[Arg^9]bradykinin from the rat LMWK fragment (Abz-MTSVIRRPPGFSPFRAPRV-NH_2). The formation of these hydrolysis products was examined in more detail by determining the kinetic parameters for the hydrolysis of synthetic, internally quenched fluorescent peptides containing six N- or C-terminal amino acids of bradykinin added to the five downstream or upstream residues of mouse and rat kininogens respectively. One of these peptides, Abz-GFSPFRAPRVQ-EDDnp (where EDDnp stands for ethylenediamine 2,4-dinitrophenyl), was preferentially hydrolysed at the Phe–Arg bond, confirming the potential des-[Arg^9]bradykinin-releasing activity of hK1 on rat kininogen. The proline residue that is two residues upstream of bradykinin in rat kininogen is, in part, responsible for this pattern of hydrolysis, since the peptide Abz-GFSPFRASRVQ-EDDnp was preferentially cleaved at the Arg–Ala bond by hK1. Since this peptidase accepts the arginine or phenylalanine residue at its S_1 subsite, this preference seems to be determined by the prime site of the substrates. These findings also suggested that the effects observed in rats overexpressing hK1 should consider the activation of B1 receptors by des-[Arg^9]bradykinin. For further comparison, two short internally quenched fluorescent peptides that bind to hK1 with affinity in the nM range and some inhibitors described previously for hK1 were also assayed with mK1 and rK1.

Key words: bradykinin, fluorescent peptide, inflammation, inhibitor, kallikrein, protease.

INTRODUCTION

Tissue kallikreins constitute a subgroup of serine proteases that share significant homology with true-tissue kallikrein, encoded by the *KLK1* gene [1,2]. Human true-tissue kallikrein, hK1 (EC 3.4.21.35), is a member of a family of 15 related serine proteases, including two enzymes expressed in prostate, namely prostate-specific antigen (hK3) and a trypsin-like enzyme hK2. These proteases share a significant degree of sequence and structural similarities and the genes are clustered together at one locus at least in three species, namely mouse, rat and human [3–6]. Despite their highly conserved structures, their known or predicted peptidase functions are specific and distinct from one another, and their wide range of expression patterns indicates their involvement in diverse physiological processes [2,7,8]. Phylogenetic analyses show that tissue kallikrein genes emerged after the separation of primate and rodent lineages; therefore, the independent evolution of tissue kallikrein genes in rodents and humans indicates that the corresponding serine proteases expressed by these genes could be involved in biological processes specific for each species [9–11]. The proteases can present peptidase specificities that are also particular for each species. Human tissue kallikrein hK1 has the best-established functions, which include release of Lys-bradykinin (kallidin) in inflammatory processes such as arthritis, asthma and rhinitis [12–14] and the processing of hormone and other peptide precursors [2,15]. hK1 releases Lys-bradykinin by limited proteolysis of high- and low-molecular-mass kininogens by cleavage at the Met^{379}-Lys^{380} and Arg^{389}-Ser^{390} bonds [16]. Rat tissue kallikrein, rK1, cleaves a Lys–Arg bond and generates bradykinin from bovine and rat kininogens [17,18], and mouse submandibular tissue kallikrein also releases bradykinin from bovine [19] and mouse [20] kininogens.

Little is known about the species specificity of tissue kallikrein–kininogen interaction and the specificity of tissue kallikrein from animals used as model for the study of the kallikrein–kinin system. The mouse model was used for assays of hK1 inhibitors [21], for knockout of mouse tissue kallikrein mK1 [22] and for overexpression of hKLK1 in a transgenic hypotensive lineage. A rat model for gene therapy of hypertension was used to assess the effects of administration of the human kallikrein gene, either in the form of naked DNA or in an adenovirus vector [23,24]. In addition, the hypotensive effect of the expression of hKLK1 was demonstrated in a transgenic rat lineage [25].

To characterize the inter-species specificities of mK1, rK1 and hK1, we describe in the present study the activities of these proteases on the synthetic peptides Abz-MTEMARRPPGFSPFRSV-TVQ-NH_2 (where Abz stands for *o*-aminobenzoyl) and Abz-MT-SVIRRPPGFSPFRAPRV-NH_2, which correspond to fragments

Abbreviations used: ACN, acetonitrile; Abz, *o*-aminobenzoyl; Aca, (*cis*, *trans*)-aminocyclohexyl-alanine; EDDnp, ethylenediamine 2,4-dinitrophenyl; hK1, mK1, rK1 and pK1, human, mouse, rat and pig true-tissue kallikreins respectively; *hKLK1*, *mKLK1*, *rKLK1* and *pKLK1*, the corresponding genes for the proteins hK1, mK1, rK1 and pK1; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; MCA, 4-methylcoumarin-7-amide; Pac, phenyl-acetyl; PKSI-527, *trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanine-4-carboxymethylanilide.

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Met^{374}-Gln^{393} and Met^{375}-Val^{393} of mouse (SwissProt accession no. 008677) and rat [27] low-molecular-mass kininogens. These two peptides span the bradykinin sequence in these kininogens. The fluorescent marker Abz was added at the N-terminal end of each peptide. Release of bradykinin from these two peptides requires the hydrolysis of Arg–Arg and Arg–Ser (or Arg–Ala) peptide bonds. The hydrolysis of these peptide bonds in mouse and rat kininogens for the release of bradykinin by mK1 and rK1 was examined in more detail by determining the kinetic parameters for the hydrolysis of internally quenched fluorescent peptides containing the six C-terminal amino acids of bradykinin added to the six upstream residues of mouse (Abz-GFSPFRSVTVQ-EDDnp, where EDDnp stands for ethylenediamine 2,4-dinitrophenyl) and rat (Abz-GFSPFRAPRVQ-EDDnp) kininogens. Similarly, two other peptides were synthesized, i.e. those containing the two N-terminal amino acids of bradykinin added to six downstream residues of mouse (Abz-MTEMARRPQ-EDDnp) and rat (Abz-MTSVIRRPQ-EDDnp) kininogens.

For further comparison with hK1, two peptidyl-MCA substrates (where MCA stands for 4-methylcoumarin-7-amide), described previously for hK1, two short internally quenched fluorescent peptides that bind to hK1 with nM affinity and some inhibitors described previously for hK1 [28–31] were also assayed with mK1 and rK1.

MATERIALS AND METHODS

Peptides

All the intramolecularly quenched fluorogenic peptides that contain EDDnp attached to glutamine residue were obtained by the solid-phase peptide synthesis method, details of which are available in [32]. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system; Shimadzu, Kyoto, Japan) was used for the solid-phase synthesis of all the peptides by the Fmoc (fluoren-9-ylmethoxycarbonyl) procedure. The short internally quenched fluorescent peptides were obtained by synthesis in solution [28]. The final deprotected peptides were purified by semipreparative HPLC using an Econosil C18 column (10 μm; 22.5 mm $\times$ 250 mm) and the following two-solvent system: solvent A, trifluoroacetic acid/water (1:1000) and solvent B, trifluoroacetic acid/ACN (acetonitrile)/water (1:900:100). The column was eluted at a flow rate of 3 or 5 ml/min with a 10–50 or 30–60 % gradient of solvent B for 30 or 45 min. Analytical HPLC was performed using a binary HPLC system (Shimadzu) equipped with an SPD-10AV Shimadzu UV–Visible detector and a Shimadzu RF-535 fluorescence detector, coupled with an Ultrasphere C18 column (5 μm, 4.6 $\times$ 250 mm), which was eluted with solvent systems A_1 (H_3PO_4/water, 1:1000) and B_1 (ACN/water/H_3PO_4, 900:100:1) at a flow rate of 0.8 ml/min and a 10–80 % gradient of B_1 for 15 min. The HPLC column eluates were monitored by their absorbance A_{220} and by fluorescence emission at 420 nm following excitation at 320 nm. The molecular mass and purity of the synthesized peptides were examined by MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS; TofSpec-E; Micromass, Manchester, U.K.) and/or peptide sequencing using a protein sequencer PPSQ-23 (Shimadzu, Tokyo, Japan). The concentrations of the substrate solutions were determined by colorimetric measurement of the Dnp (2,4-dinitrophenyl) group (molar absorption coefficient of 17 300 $M^{-1} \cdot cm^{-1}$ at 365 nm).

Enzymes

Mouse true-tissue kallikrein mK1 (originally reported as proteinase F [33] and later identified as mK1 [19]) was purified from the submandibular glands of 52 female mice (ICR strain) by the procedure described in [33]. The purified enzyme had a specific activity similar to that reported previously and was 90–95 % pure when it was analysed by reverse-phase HPLC. The purified enzyme was dissolved in 1 mM HCl and stored at -80 °C with minimum repetition of freezing and thawing. Homogeneous preparations of human tissue kallikrein, obtained by the method of Shimamoto et al. [34], and rat tissue kallikrein, obtained as described previously [35], were kindly provided by Dr J. Chao (Medical University of South Carolina, Charleston, U.S.A.). The molar concentrations of enzyme solutions were determined by active-site titration with 4-methylumbelliferyl-*p*-guanidinobenzoate [36].

Enzyme hydrolysis of fluorescent-quenched substrates

Kinetic assays

Fluorogenic peptidyl substrates were hydrolysed under the following conditions: 20 mM (for hK1) or 50 mM (for rK1 and mK1) Tris/HCl and 1 mM EDTA, pH 9 (for hK1) or pH 8.5 (for rK1 and mK1), at 37 °C; the reactions were monitored by measuring the fluorescence changes at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm in a Hitachi F-2500 spectrofluorimeter. The 1 cm pathlength cuvette containing 1 ml of buffer solution + substrate was placed in a thermostatically controlled cell compartment for 5 min before the addition of the enzyme solution and the increase in fluorescence with time was continuously recorded for 10 min. The slope was converted into mol of hydrolysed substrate/min based on the fluorescence curves of standard peptide solutions before and after total enzyme hydrolysis. The concentration of the peptide solutions was determined by colorimetric measurement of the Dnp group (molar absorption coefficient at 365 nm of 17 300 $M^{-1} \cdot cm^{-1}$). The enzyme concentration for the initial rate determination was chosen at a level intended to hydrolyse less than 5 % of the substrate present. The kinetic parameters were calculated by the method of Wilkinson [37] as well as by using Eadie–Hofstee plots. All the results obtained were fitted to non-linear least-squares equations using Grafit version 3.0 from Erithacus Software (Horley, Surrey, U.K.). The S.D. of K_m and k_{cat} determinations were in no case higher than 6 % of the obtained value.

Determination of the cleavage point in the assayed substrates

The cleaved bonds were identified by isolation of the fragments by HPLC and the retention times of the fluorescent Abz-containing fragments were compared with authentic synthetic sequences and/or by amino acid sequencing. All the fragments were also checked by MALDI–TOF-MS.

RESULTS AND DISCUSSION

Release of kinins from the bradykinin-containing peptide fragments of mouse and rat kininogens by mK1, rK1 and hK1

The Met^{374}–Gln^{393} fragment of mouse kininogen (Abz-MTEMARRPPGFSPFRSVTVQ-NH_2) was hydrolysed by mK1 first at the Arg–Ser bond, accumulating the fragment Abz-MTEMARRPPGFSPFR, which was then hydrolysed at the Arg–Arg bond at a lower rate with the release of bradykinin (RPPGFSPFR). Figure 1 shows the time course of these reactions as monitored by HPLC and MS. The fragment Met^{375}–Val^{393} of rat kininogen (Abz-MTSVIRRPPGFSPFRAPRV-NH_2) was hydrolysed by rK1 in a similar pattern; the Arg–Ala bond was hydrolysed quickly, and the Arg–Arg bond in Abz-MTSVIRRPPGFSPFR was cleaved more slowly to release bradykinin (see Figure 2). The rat kininogen fragment was also hydrolysed by mK1, producing bradykinin

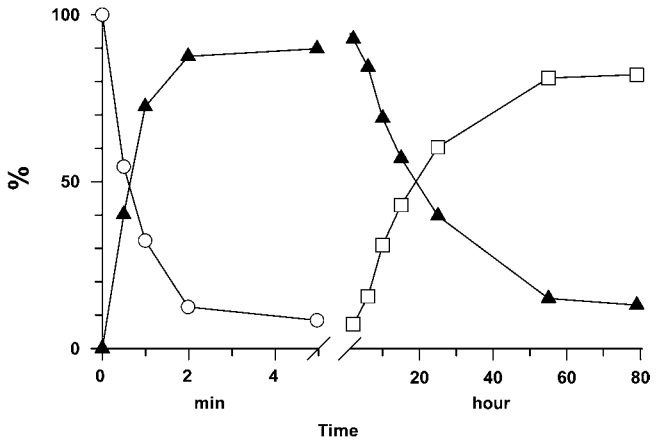

Figure 1 Time course of hydrolysis by mK1 of the mouse kininogen fragment, Abz-MTEMARRPPGFSPFRSVTVQ-NH_2

Each product was isolated and characterized by MALDI–TOF-MS. (○) Abz-MTEMARRPPGFSPFRSVTVQ-NH_2 is the substrate that is rapidly hydrolysed forming (▲) Abz-MTEMARRPPGFSPFR, which is slowly hydrolysed to (□) Abz-MTEMAR or bradykinin (superimposed). The substrate and their products of hydrolysis were quantified by HPLC and are shown as percentage of product formation or substrate degradation. Analytical HPLC conditions were: an Ultrasphere C18 column (5 μm, 4.6 × 250 mm) which was eluted with the solvent systems A_1 (H_3PO_4/water, 1:1000) and B_1 (ACN/water/H_3PO_4, 900:100:1) at a flow rate of 0.8 ml/min and a 10–80 % gradient of B_1 for 15 min. The HPLC column eluates were monitored by their A_{220} and by fluorescence emission at 420 nm following excitation at 320 nm. Hydrolysis conditions were: 50 mM Tris/HCl (pH 8.5), 1 mM EDTA, 37 °C, [mK1] = 64 nM, [substrate] = 37 μM.

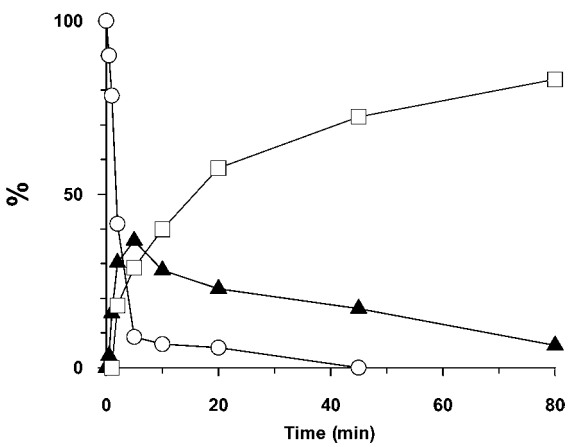

Figure 2 Time course of hydrolysis by rK1 of the rat kininogen fragment Abz-MTSVIRRPPGFSPFRAPRV-NH_2

Each product was isolated and characterized by MALDI–TOF-MS. (○) Abz-MTSVIRRPPGFSPFRAPRV-NH_2 is the substrate that is rapidly hydrolysed forming (▲) Abz-MTSVIRRPPGFSPFR, which is slowly hydrolysed to (□) Abz-MTSVIR or bradykinin (superimposed). All the peptides were quantified by HPLC (conditions are described in the legend to Figure 1) and are shown as percentage of their formation or substrate degradation. Hydrolysis conditions were: 50 mM Tris/HCl (pH 8.5), 1 mM EDTA, 37 °C, [rK1] = 7 nM, [substrate] = 37 μM.

with a similar time course (results not shown). hK1 released bradykinin from the mouse kininogen fragment (Abz-MTEMARRPPGFSPFRSVTVQ-NH_2), but preferentially released des-[Arg^9]bradykinin from rat kininogen (Abz-MTSVIRRPPGFSPFRAPRV-NH_2) as shown in Figure 3. We were surprised to find that hK1 preferentially cleaved the Phe–Arg bond compared with the Arg–Ala bond, resulting in the formation of higher amounts of des-[Arg^9]bradykinin than bradykinin. This observation was

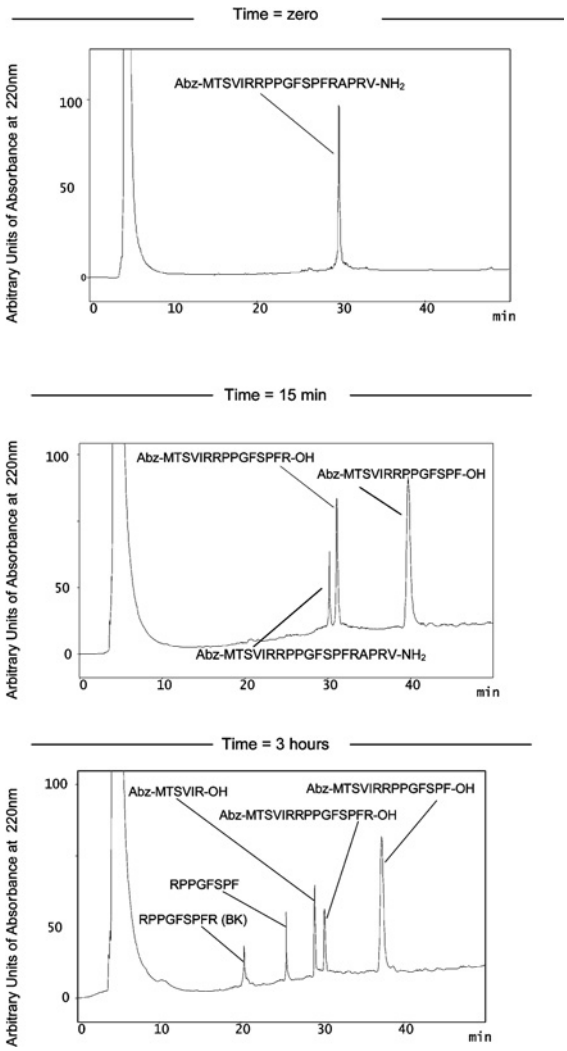

Figure 3 Time course of hydrolysis by hK1 of the rat kininogen fragment Abz-MTSVIRRPPGFSPFRAPRV-NH_2

Each peak was isolated and characterized by MALDI–TOF-MS. Only the UV recording is presented. Top panel: reaction time = time zero; only the UV peak of Abz-MTSVIRRPPGFSPFRAPRV-NH_2 was detected. Middle panel: reaction time = 15 min; the UV peaks of the products of hydrolysis Abz-MTSVIRRPPGFSPFR-OH and Abz-MTSVIRRPPGFSPF-OH and the small amount of residual substrate were detected. Bottom panel: reaction time = 3 h; Abz-MTSVIR-OH, RPPGFSPFR (bradykinin, BK) and RPPGFSPFR (des-[Arg^9]bradykinin) and the residual fragments Abz-MTSVIRRPPGFSPFR-OH and Abz-MTSVIRRPPGFSPF-OH of the first hydrolysis were detected. Hydrolysis conditions were: 20 mM Tris/HCl (pH 9.0), 1 mM EDTA, 37 °C, [hK1] = 40 nM and [substrate] = 20 μM. HPLC conditions were the same as described in Figure 1.

confirmed by the preferred cleavage of the Phe–Arg bond by hK1 in the peptide Abz-GFSPFRAPRVQ-EDDnp (Table 1), an internally quenched fluorescent peptide containing the ten C-terminal amino acids of Abz-MTSVIRRPPGFSPFRAPRV-NH_2. Hydrolysis at the Phe–Arg bond by hK1 in both peptides was in accordance with an earlier observation of efficient cleavage after phenylalanine in peptides derived from the reactive-centre loop of kallistatin [38], which is a specific serpin for hK1, and in somatostatin [29]. Therefore hK1 is not a restricted arginyl hydrolase despite its structural similarities to trypsin. In addition, the hydrolysis at the Phe–Arg bond indicates the acceptance by hK1 of proline residue at its S_2 subsite; this subsite has previously been demonstrated to accommodate the phenyl side chain of

Table 1 Kinetic parameters for hydrolysis by mK1, rK1 and hK1 of internally quenched fluorescent peptides derived from mouse and rat kininogens and a short quenched fluorescent substrate containing a pair of basic amino acids

Conditions of hydrolysis: 20 mM (for hK1) or 50 mM (for rK1 and mK1) Tris/HCl and 1 mM EDTA, pH 9 (for hK1) or pH 8.5 (for rK1 and mK1), at 37°C.

Protease	mK1	rK1	hK1	mK1	rK1	hK1
Mouse sequence	Abz-GFSPFR↓SVTVQ-EDDnp			Abz-MTEMAR↓RPQ-EDDnp		
k_{cat} (s^{-1})	7.0	14.7	3.1	1.49	0.8	0.73
K_m (μM)	0.17	2.51	2.1	3.0	6.16	7.35
k_{cat}/K_m	41176	5880	1485	496	130	99
Rat sequence	Abz-GFSPFR↓APRVQ-EDDnp			Abz-MTSVIR↓RPQ-EDDnp		
k_{cat} (s^{-1})	6.6	12.0	*	3.27	5.86	Slow hydrolysis
K_m (μM)	0.96	1.47	*	2.40	2.23	Slow hydrolysis
k_{cat}/K_m	6948	8163	*	1362	2663	Slow hydrolysis
Short IQF peptide	Abz-FR↓R-EDDnp			Abz-FR↓K-EDDnp		
k_{cat} (s^{-1})	12.7	18.7	Slow hydrolysis†	18.4	21.8	Slow hydrolysis‡
K_m (μM)	0.33	0.2	Slow hydrolysis†	0.77	0.24	Slow hydrolysis‡
k_{cat}/K_m	38 697	93 500	Slow hydrolysis†	23 896	90 916	Slow hydrolysis‡

* The peptide Abz-GFSPFRAPRVQ-EDDnp was cleaved at two peptide bonds, R↓A (30 %) and F↓R (70 %) with $k_{cat}/K_m = 4545$.
† $K_i = 20$ nM.
‡ $K_i = 74$ nM.

phenylalanine very well [39], which has been considered as a requisite for a good substrate for tissue kallikreins [40]. The shift of the cleavage site by hK1 from the Arg–Ala to the Phe–Arg bond in rat kininogen peptides is probably due to the presence of the two proline residues upstream of bradykinin in rat kininogen. This directional determinant effect of proline residue can also explain the release by hK1 of Lys-bradykinin instead of bradykinin from human kininogen. In this protein, the sequence at the N-terminal site of bradykinin is . . . ISLMKRPP . . .; therefore, for the release of bradykinin, the Lys–Arg bond should be cleaved; however, the presence of proline residue at the prime site of arginine residue shifts the cleavage to the Met–Lys bond and the release of Lys-bradykinin. The efficiency of hK1 in cleaving the Met–Lys bond in peptides spanning the N-terminal side has previously been shown to be significantly influenced by the $P_1' - P_3'$ substrate positions [41]. Consistent with this result, the hydrolysis of the peptides Abz-GFSPFRAPRVQ-EDDnp and Abz-GFSPFRASRVQ-EDDnp, presented in Figure 4 and discussed below, further supports the effect of proline at P_2' on the determination of the site of cleavage, in this case at the Phe–Arg or Arg–Ala bond. The preferred release of des-[Arg^9]bradykinin by hK1 instead of bradykinin from the synthetic rat kininogen fragment suggested that the cardiovascular effects observed in rat overexpressing human kallikrein as models of gene therapy of hypertension [23–25] should consider the activity of des-[Arg^9]bradykinin on B1 receptors. Although we did not use purified rat kininogens to verify the release of des-[Arg^9]bradykinin due to the significant large instability of these proteins, the synthetic peptides derived from the segments of kininogen that contain bradykinin sequence have been useful in characterizing the kininogenase activity of tissue kallikreins such as hK1 [42], pK1 [43] and hK2 [44]. Previously, we used these kinds of peptides to characterize the kininogenase activities of cysteine proteases from *Trypanosoma cruzi* (cruzipain) [45] and *Fasciola hepatica* [46], enzymes that do not belong to the tissue kallikrein family.

Kinetic parameters for the hydrolysis of fluorescent peptides

To evaluate the kinetic parameters for the hydrolysis of the peptide bonds cleaved by mK1, rk1 and hK1 in the mouse and rat kininogen synthetic fragments described above, the internally quenched fluorescent peptides Abz-MTEMARRPQ-EDDnp, Abz-GFSPFRSVTVQ-EDDnp, Abz-MTSVIRRPQ-EDDnp and Abz-GFSPFRAPRVQ-EDDnp were synthesized and assayed with the three kallikreins. As shown in Table 1, mK1 and rK1 hydrolysed these peptides at the same bonds as cleaved in the bradykinin-containing fragments of mouse and rat kininogens described above. The peptides Abz-GFSPFRSVTVQ-EDDnp and Abz-GFSPFRAPRVQ-EDDnp were hydrolysed more efficiently compared with Abz-MTEMARRPQ-EDDnp and Abz-MTSVIRRPQ-EDDnp by both mK1 and rK1 (Table 1), which is in accordance with the time course for hydrolysis of the bradykinin-containing fragments of mouse and rat kininogens (Figures 1 and 2). mK1 hydrolysed Abz-GFSPFRSVTVQ-EDDnp (mouse sequence)

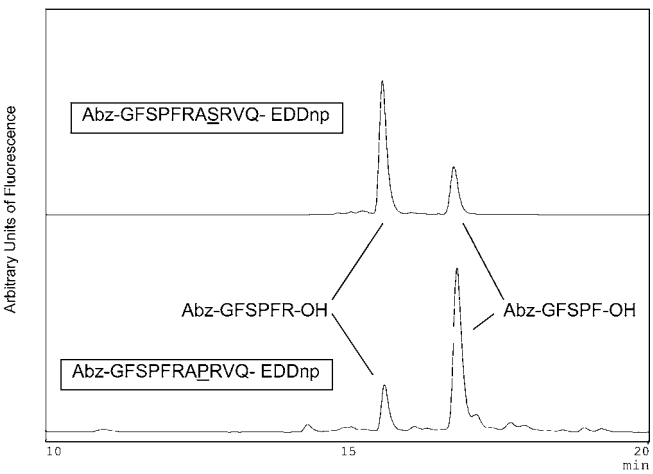

Figure 4 HPLC profile of the fluorescent products of complete hydrolysis by hK1 of the peptides Abz-GFSPFRAPRVQ-EDDnp and Abz-GFSPFRASRVQ-EDDnp

The starting substrates were completely hydrolysed after 1 h of incubation with hK1 under the same conditions as described in Figure 3. Hydrolysis of Abz-GFSPFRASRVQ-EDDnp resulted in a larger peak of Abz-GFSPFR-OH compared with the hydrolysis of Abz-GFSPF-OH, and the inverse was observed with the hydrolysis of Abz-GFSPFRAPRVQ-EDDnp.

with higher k_{cat}/K_m values when compared with rK1 or hK1, indicating a clear species specificity. Hydrolysis of the peptides Abz-MTEMARRPQ-EDDnp and Abz-MTSVIRRPQ-EDDnp was also species-specific, since the respective k_{cat}/K_m values are higher for the substrate sequence derived from the kininogen of the same species, although they were poorer substrates for all the three assayed kallikreins. Sueyoshi et al. [20] reported a higher kininogenase activity of mK1 compared with other kallikreins using a low-molecular-mass mouse kininogen isolated from the ascitic fluid of sarcoma 180 tumour-bearing mice as the substrate. Sueyoshi et al. [20] suggested that this particular species specificity of mK1 was based on its ability to hydrolyse Boc-MAR-MCA (where Boc stands for t-butoxycarbonyl), which contains the three amino acids immediately downstream of the bradykinin sequence in mouse kininogen, with k_{cat}/K_m values approx. 100 times higher than that of pK1. Our results did not show such a large difference for the hydrolysis by rodent kallikreins of the Arg–Arg bond on the N-terminal side of bradykinin compared with the efficient hydrolytic activity on the Arg–Ser bond at the C-terminal site of bradykinin. It is noteworthy that the capacity of mK1, rK1 and hK1 to hydrolyse Abz-MTEMARRPQ-EDDnp at the Arg–Arg bond is probably because the enzyme prefers an alanine residue at the S_2 subsite; in contrast, as mentioned above, the S_2 subsites of hK1 and pK1 have been described to have a restricted preference for hydrophobic, particularly for aromatic amino side chains [40,47,48]. The potential ability of hK1 to release des-[Arg^9]bradykinin from rat kininogen was confirmed by the activity of this enzyme on the peptide Abz-GFSPFRAPRVQ-EDDnp, which was hydrolysed mainly at the Phe–Arg bond. Analysis of the products of hydrolysis by hK1 analogue of this peptide, Abz-GFSPFRASRVQ-EDDnp, where a proline was substituted with a serine residue, showed that hK1 hydrolysed preferentially at the Arg–Ala bond (Figure 4). The k_{cat}/K_m value for the hydrolysis of this peptide by hK1 was 32 323, which is almost one order of magnitude higher than that for the hydrolysis of Abz-GFSPFRAPRVQ-EDDnp by the same enzyme. This result is further evidence for the role of the proline residue on the prime side of the substrates in determining both the efficiency and the peptide bond to be cleaved by hK1 that accepts arginine or phenylalanine at the S_1 subsite.

We further compared the reactivity of hK1 with mK1 and rK1, examining their hydrolytic activity on two short internally quenched fluorescent peptides containing a pair of basic amino acids (Abz-FRR-EDDnp and Abz-FRK-EDDnp), which were described previously for use with cysteine and serine proteases [28]. These peptides were hydrolysed by hK1 with very low velocity, but bound to the enzyme with K_i values in the nM range. They probably represent model peptides for designing specific and potent inhibitors for hK1. The high k_{cat}/K_m values for the hydrolysis of these two peptides by rK1 and mK1 as compared with the very slow hydrolysis by hK1 (Table 1) was surprising. These results indicate that rK1 and mK1 have a particular preference for hydrolysis between basic amino acids, an additional characteristic of rodent kallikreins that provides them with the ability to release bradykinin instead of Lys-bradykinin as observed with kallikreins of other species.

We have previously described the efficiency of hK1 in hydrolysing substrates with phenylalanine at the P_1 position [29,38] and observed that the small peptide pFF-MCA (where p stands for proline in D-diastereo-isomer form) was quite specific for hK1. To examine this further, the hydrolytic activities of mK1 and rK1 on pFF-MCA were compared. Poor hydrolysis was observed with both enzymes, as indicated by the low values of k_{cat} (Table 2). However, the low K_m values for the hydrolysis of this peptide by mK1 and rK1 are noteworthy, suggesting that these enzymes can accommodate this peptide, although in an unproductive way, for catalysis. It appears that the phenylalanine residue at the P_1 position does not fit the S_1 subsite of mK1 or rK1 as in hK1, which was confirmed by the very efficient hydrolysis of pFR-MCA (an excellent substrate for the rodent kallikreins, particularly for rK1; Table 2).

Table 2 Kinetic parameters for hydrolysis by mK1, rK1 and hK1 of the fluorescent peptides pFR-MCA and pFF-MCA

Conditions of hydrolysis were the same as described in Table 1.

Protease	mK1	rK1	hK1	mK1	rK1	hK1
Peptidyl-MCA	pFR-MCA			pFF-MCA		
k_{cat} (s^{-1})	3.18	95.3	3.6	0.085	0.05	12.5
K_m (μM)	0.18	1.5	3.1	2.7	1.0	70
k_{cat}/K_m	17 666	63 553	1161	30.7	50	179

Table 3 Inhibition constant (K_i) of the previously described inhibitors of hK1 for mK1 and rK1

	K_i (μM)			
Peptide	rK1	mK1	hK1	Reference
Pac-FSR-EDDnp	3.5 ± 0.1	4.2 ± 0.04	0.70 ± 0.05	[31]
Pac-Aca-SR-NH_2	16.9 ± 1.0	12.1 ± 0.01	0.11 ± 0.01	[30]
Ac-kFFPLE-NH_2	4.6 ± 0.2	No inhibition	0.029 ± 0.004	[49]
PKSI-527	347 ± 12	48.1 ± 0.08	> 800	

Inhibition of mK1 and rK1 by inhibitors of hK1

We have described previously inhibitors of hK1 and those that functioned at the nM range [30,31,49] were assayed as inhibitors of mK1 and rK1. Table 3 shows the K_i values obtained for them with mK1 and rK1; note that the kinetics of inhibition was of the competitive type. Pac-FSR-EDDnp (where Pac stands for phenylacetyl) inhibited hK1 with a K_i value five times lower than that for mK1 or rK1. However, this peptide was demonstrated to have significant inhibitory effects on nociceptive transmission and inflammatory responses in mice [21]. The peptide Pac-Aca-SR-NH_2 [where Aca stands for (*cis*, *trans*)-aminocyclohexyl-alanine] was described previously as a member of a series of peptides specially designed to introduce non-natural basic amino acids at the S_1 subsite of hK1 [49]. Pac-Aca-SR-NH_2 was resistant to hydrolysis by hK1, mK1 and rK1, but the K_i values for the inhibition of the last two enzymes were two orders of magnitude higher than that for hK1. This result indicates that mK1 and rK1 do not accept amino acids bearing a large hydrophobic side chain at their S_1 subsites and do not accept even those bearing a positive primary amino group in their structure.

The peptide Ac-kFFPLE-NH_2 is the best inhibitor of hK1 as shown in Table 3, but it did not inhibit mK1 at a concentration below 50 μM, and the K_i value for rK1 is two orders of magnitude higher. Surprisingly, the peptide Ac-kFFPLE-NH_2 also inhibited human plasma kallikrein, with a K_i value of 8 nM. This enzyme is known as a restricted arginyl hydrolase. A specific inhibitor of human plasma kallikrein, PKSI-527 (*trans*-4-aminomethylcyclohexenecarbonyl-L-phenylalanine-4-carboxymethylanilide), was described previously [50]. Its *trans*-4-aminomethylcyclohexenecarbonyl moiety is supposed to interact with the

negatively charged S_1 subsite of human plasma kallikrein, resulting in the specificity of the inhibitor. PKSI-527 was assayed against mK1 and rK1 and the K_i values obtained show that it is a poor inhibitor of these enzymes. Although mK1 and rK1 are preferentially arginyl hydrolases, similar to plasma kallikrein, the interactions of mK1 and rK1 with PKSI-527 were clearly very unfavourable. The *trans*-4-aminomethylcyclohexenecarbonyl moiety of this inhibitor is similar to that of Aca in the peptide Pac-Aca-SR-NH_2 discussed above, which was also a poor inhibitor of mK1 and rK1. The inhibitory activity of the peptide Ac-kFFPLE-NH_2 on hK1 has been interpreted as mainly due to the presence of glutamic residue at the S_3' position [30]. This result suggests that the negative side chain of glutamic residue could play the same role as the EDDnp, the NO_2 groups of which introduce a strong polarization into this group, generating a negative moiety that could interact favourably with hK1.

In conclusion, the use of mice or rats as animal models for studying the kallikrein–kinin system has to take into account the difference in specificity between rodent enzymes and enzymes of other mammals, particularly hK1 that can release des-[Arg^9] bradykinin from rat kininogen due to its acceptance of phenylalanine besides arginine residue at the S_1 subsite. Inhibitors that were found to inhibit hK1 in the nM range are weak inhibitors of mK1 or rK1. The differences described in the present study could be due to the evolution of rodent glandular kallikreins [9], which emerged after the separation of primate and rodent lineages and can result in biological processes specific for each species.

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